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## **Differentiation of mouse and human ES cells into hepatic lineages**

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Running title: Turning ES cells into liver

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## SUMMARY

We recently reported a novel method to induce ES cells differentiate into an endodermal fate, especially pancreatic, using a supporting cell line. Here we describe the modified culture condition with the addition and withdrawal of secreted growth factors could induce ES cells to selectively differentiate into a hepatic fate efficiently. The signaling of BMP and FGF that have been implicated in hepatic differentiation during normal embryonic development are demonstrated to play pivotal roles in generating hepatic cells from the definitive endoderm derived from ES cells. Moreover, the expression of AFP, Albumin or a biliary molecular marker appeared sequentially thus suggested the differentiation of ES cells recapitulated normal developmental processes of liver. The ES cell-derived differentiated cells demonstrated evidence of glycogen storage, secreted Albumin, exhibited drug metabolism activities and expressed a set of cytochrome or drug conjugate enzymes, drug transporters specifically expressed in mature hepatocytes. With the same procedure, human ES cells also gave rise to cells with mature hepatocytes' characteristics. In conclusion, this novel procedure for hepatic differentiation will be useful for elucidation of molecular mechanisms of hepatic fate decision at gut regionalization, and could represent an attractive approach for a surrogate cell source for pharmaceutical studies such as toxicology.

## INTRODUCTION

Liver performs many complex functions including carbohydrate metabolism, urea and lipid metabolism, storage of essential nutrients, production and secretion of bile acids, into which metabolites of drugs and other compounds are excreted. Hepatic biotransformation of a drug involves activations of inactive prodrugs to active drugs, inactivations, detoxifications as well as adverse activations, where some metabolites are more toxic than the parent drug molecules. Therefore, the examination of drug biotransformation using human liver cells plays an important role in the early stages of the drug discovery process. However, primary liver cells are short-lived and cannot be maintained in culture for long term. Considerable donor dependent variations are also problematic. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocysts. They apparently differentiate into various types of mature cells, thereby are attractive source for routine access to large numbers of cells that can be used for the development of new drug-screening strategies instead of primary cells (Davila *et al.* 2004; Kulkarni & Khanna 2006).

The vertebrate liver develops from the ventral foregut endoderm (Wells & Melton 1999; Zaret 2000). Although several lines of evidences have shown that different regions of the gut are patterned by signals from the underlying mesoderm, the mechanism of how specific endoderm tissues differentiate remains much unknown. In hepatic development, Fibroblast growth factors (FGF) and Bone morphogenetic proteins (BMP) originated from the cardiac mesoderm and the septum transversum mesenchyme are shown to be essential for cardiac induction, promotion of liver gene expression and endoderm proliferation (Jung *et al.* 1999; Rossi *et al.* 2001). FGF signaling from the cardiac mesoderm directs the overlying ventral endoderm to adopt a hepatic fate instead of a pancreatic fate (Deutsch *et al.* 2001).

Several studies reported the induction of ES cell differentiation into hepatic cells both *in vitro* and *in vivo*. The *in vitro* approaches involve the formation of embryoid bodies to mimic the

inductive microenvironment required for liver organogenesis (Asahina *et al.* 2004; Heo *et al.* 2006) or treatment with specific growth factors and cytokines critical for hepatocyte differentiation (Teratani *et al.* 2005). Also the co-cultivation of ES cells with embryonic mesenchymal cells was shown to direct ES cells toward a hepatic lineage (Ishii *et al.* 2005; Saito *et al.* 2006; Soto-Gutierrez *et al.* 2006). Recently, *in vitro* generation of ES cell-derived hepatic cells was reported by using BMP4 (Gouon-Evans *et al.* 2006). Although these findings are encouraging, there are few studies for fate divergence from gut endoderm into liver, pancreas and intestine cells. Hepatic differentiation of ES cells in most studies was not efficient for producing enough amounts of functional mature hepatocytes.

We have established a novel procedure to direct ES cells differentiate into regional specific gut endoderm lineages, such as liver, pancreas, lung or intestine, by co-cultivation with a supporting mesoderm-derived cell line, M15, with selective culture conditions. Using this novel procedure, the present study documents an efficient induction of ES cells into morphologically, and biologically functional hepatocytes. Moreover, this method was applicable to human ES cells. Under selective culture conditions, approximately 80% of the human ES cells can be manipulated to differentiate into AFP-positive cells, and 9% of total cells turned to Albumin-positive cells. Analysis of the hepatic cells derived from ES cells showed that these cells resemble mature hepatocytes in that they store glycogen, and expressed various molecular markers such as Albumin, bile acid transporters, cytochrome P450 metabolic enzymes.

## RESULTS

### **M15, a mesoderm-derived cell line, directs ES cells to differentiate into endodermal and hepatic lineages**

We have recently established a co-cultivation method using a mesoderm-derived cell line, M15. ES cells grown on M15 and treated with Activin and bFGF during the first 4 days of differentiation, are sequentially directed to differentiate into mesendoderm and then into definitive endoderm. A prolonged addition of Activin and bFGF on d4-8 specifically increased pancreatic differentiation marked by an increase in *Pancreatic and duodenal homeobox gene 1* (*Pdx1*)-expressing cells, while the withdrawal of Activin and bFGF induced  $\alpha$ -fetoprotein (*Afp*) expression (Shiraki *et al.* 2008).

In an attempt to find a condition in which ES cells dominantly adopt a hepatic fate instead of a pancreatic fate, Activin and bFGF were added to ES cells grown on M15 on d0-4, and then withdrawn. On d4-8, various soluble factors were examined, in combination with media supplemented with FBS or KSR, with varied glucose concentrations (2000 mg/L or 4500 mg/L). Hepatocyte growth factor (HGF), Oncostatin M (OsM), or dexamethasone (Dex) were included since these were reported to potentiate hepatic differentiation of ES cells (Michalopoulos *et al.* 2003; Teratani *et al.* 2005).

On day 8, both definitive endoderm and pancreatic cells were quantified by flow cytometry using E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup> (Yasunaga *et al.* 2005), and Pdx1 as markers, respectively. To quantify Pdx1 expression, we used an ES cell line, SK7, established from a transgenic mouse line P#48.9 (Gu *et al.* 2004) bearing a Green fluorescent protein (GFP) reporter driven by the promoter for *Pdx1* (Shiraki *et al.* 2008). Figure 1A shows that the proportion of both the E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup> cells and GFP<sup>+</sup> (Pdx1-expressing) cells increased when Activin and bFGF were added on d4-8 (Fig 1A, filled bars). The addition of Dex, HGF and OsM decreased the proportion of E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup> cells as well as Pdx1-expressing cells (Fig. 1A).

To define the extent of hepatic differentiation within gut definitive endoderm, we quantified transcript levels for *Afp*, an early hepatic marker (Shiojiri 1981), and *Sonic hedgehog* (*Shh*), which is expressed in the foregut region but excluded in the pancreatic and hepatic buds (Burke & Oliver 2002)(Fig. 1B). *Afp* was induced under KSR, but not FBS condition. Moreover, under low glucose condition, and with the presence of Dex, HGF and OsM, the definitive endoderm cells turned out to give a maximum expression of *Afp* (gray bar in 2000KSR). When Activin and bFGF are added, a pancreatic fate is favored, shown by an increase in *Pdx1*/GFP positive cells (Fig. 1A, black bars), and a decrease in *Shh* and *Afp* transcripts (Fig. 1B, black bars). Conversely, when Dex, HGF and OsM were added, a non-pancreatic fate is favored (Fig. 1A, 1B, gray bars).

Immunocytochemistry (ICC) further confirmed that culture conditions for the generation of Pdx1- and AFP-expressing cells are mutually complementary and most of them arose from distinct colonies (Fig. 2A). Continuous addition of Activin and FGF yielded Pdx1-expressing cells dominantly (Activin & bFGF columns). Withdrawal of these factors followed by the addition of Dex, HGF and OsM in medium supplemented with a lower glucose concentration of 2000 mg/L and KSR instead of FBS yielded a substantial number of AFP expressing cells in the expense of Pdx1-expressing cells (2000KSR, Dex&HGF&OsM). The present result that hepatic differentiation is enhanced under serum free condition agrees well with previous reports(Kubo *et al.* 2004; Heo *et al.* 2006).

Either the single or the combinatory additions of Dex, HGF and OsM were examined with medium supplemented with KSR and lower glucose (2000 mg/L). Figure 2B shows that the effects of promoting AFP expression is in an order of Dex > HGF > OsM. Addition of Dex and HGF was enough to result in a maximum potentiation of ES cell differentiation into AFP-expressing cells.

Therefore, for maximum definitive endoderm differentiation, the following procedure was adopted: ES cells are cultured for 4 days (d0-d4) in a medium with Activin , bFGF, FBS, and

high glucose (4500 mg/L), then the medium is switched to that with KSR, low glucose (2000 mg/L) and various growth factors and their inhibitors of interest.

### **BMP and FGF signals are pivotal for hepatic differentiation of the definitive endoderm from ES cells**

Since it is reported that BMP and FGF signaling are involved in hepatic differentiation (Jung *et al.* 1999; Rossi *et al.* 2001), their effects on definitive endoderm cells were tested (Fig. 2C, D). Addition of bFGF increased AFP-positive cells, whereas addition of SU5402, a specific inhibitor of FGF receptor I, decreased AFP-positive cells. Noggin addition abolished most of the AFP-expressing cells, which was recovered by bFGF addition (Fig. 2C, D). These results demonstrate that BMP signaling is required in the induction of hepatocyte differentiation, while FGF signaling potentiates BMP signal.

### **Chronological analysis revealed a sequential induction of molecular markers demonstrating the differentiation of hepatoblasts and hepatocytes, and cells of the bile duct cell lineages.**

The cultures were then extended up to d30 and the chronological expressions of hepatic molecular markers were studied. Figure 3A shows that AFP single positive cells first appeared, and then Albumin-positive cells are detected on d18. On d18, there are cells expressing both AFP and Albumin. Later on, the proportion of AFP-expressing cells decreased, and Albumin-expressing cells increased. On d8 and d30, the proportions of AFP-positive cells were 33.1% and 7.4%, respectively (Supplementary Table 2). The results that AFP and Albumin are differentially regulated suggest that the ES cells differentiate to immature hepatic precursors (AFP+/Albumin-), then hepatoblast (AFP+/Albumin+), and then hepatocyte (AFP-/Albumin+), which appears to be coincident with the development of hepatic lineages in mouse embryos (Shiojiri 1981, 1984).



Since the hepatoblasts are bipotential progenitors capable to differentiate into hepatocyte and bile duct lineages, we then assayed for the generation of bile duct lineage in the culture. ICC analysis on d30 showed that cells of the hepatocyte lineage and bile duct lineage segregated and formed distinct colonies of Albumin+ (17.8%) or *Dolichos biflorus agglutinin* (DBA)+ (72.9%) (Takahashi *et al.* 1995)(Fig. 3B, Supplementary Table 2).

At the same time, ICC analysis of mature molecular markers showed that some of the Albumin-positive cells expressed mature hepatic markers such as  $\alpha$ -1-Antitrypsin, and two members of the cytochrome P450 (Cyp) enzymes Cyp3A or Cyp7A1 (Fig. 3C).  $\alpha$  1-Antitrypsin (Travis & Salvesen 1983; Potempa *et al.* 1994) is a serine protease and trypsin inhibitor, Cyp3A is a member of the cytochrome P450 subfamilies involved in drug and steroid metabolism (Wrighton & Stevens 1992), Cyp7A1 is a cholesterol 7 alpha hydroxylase and regulates the pathway through which cholesterol is converted into bile acids. Cyp7A1 is expressed in the liver and not in the yolk sac tissue, and thus serves as a good marker for hepatocytes (Asahina *et al.* 2004).

### **Functional characterization of the ES cell-derived hepatic cells**

To define the extent of maturation of the hepatic cells, real-time PCR analysis of hepatic molecular markers were carried out on d10 and d30 differentiated ES cells (Fig. 4). The expression levels of the molecular markers were compared with those in E12.5 fetal liver or adult liver. At E12.5, fetal liver also acts as hematopoietic tissue and is rich in hematopoietic cells. CD45-/Ter199- (double negative) E12.5 fetal liver cells were recovered and subjected to real-time PCR analysis (Fig. 4, FL). *Afp* transcripts decreased at day 30, which agrees well with the above ICC data (Fig. 3, Supplementary Table 2). Transcripts of *Alb1*, *Keratin 7* (*Cytokeratin 7*; *Krt 7*), *Keratin 19* (*Cytokeratin 19*; *Krt19*), or mature markers of cytochrome P450 enzyme *Cyp7a1*, *Cyp2b10*, *Cyp3a11*, *Cyp3a13*, *hydroxysteroid sulfotransferase* (*Sult2a1*), *UDP-glucuronosyltransferase* (*Ugt1a1*), *organic anion transporting polypeptides* (*Slco1a4*) or

*bile salt export pump (Abcb11)* (Choudhary *et al.* 2003; Wagner *et al.* 2005) were also examined (Fig. 4). These mature hepatocyte markers were detectable at d10, which increased to a substantial level at d30 of differentiation. Most of the expression levels of the markers were comparable to those of the fetal liver. *Ugt1a1*, *Cyp7a1*, *Cyp2b10*, *Cyp3a13* and *Slco1a4* expressions were higher than those of fetal liver, but lower than those of adult liver. These results thereby demonstrate that the ES cell-derived differentiated cells expressed many of the mature hepatocyte markers.

Next we performed functional studies to characterize the ES cell-derived hepatic cells. In mouse embryo, a small proportion of hepatocytes begin to accumulate glycogen in the cytoplasm from E15.5, and then hepatocytes rich in glycogen increased in number in the liver of E16.5 and E17.5 (Shiojiri 1981). Most cells on d30 of differentiation were positive for Periodic acid Schiff (PAS) staining indicative of glycogen storage, a characteristic of mature hepatocytes (Fig. 5A, d30).

Since OsM is reported to induce maturation of mouse fetal hepatocytes (Kamiya *et al.* 2001; Kamiya *et al.* 2002), the effect of OsM on Albumin secretion was assayed with an enzyme-linked immunosorbent assay (ELISA). Figure 5B showed that the Albumin secretion was not detected at d8 when hepatoblast-like (AFP+/Albumin+) cells appeared (Fig. 3). The potentiation effect of OsM on Albumin secretion was not observed until d40 of differentiation. At d50 and d60, a two-fold increase in Albumin secretion was detected in OsM, Dex and HGF supplemented differentiation media compared to control media supplemented with Dex and HGF.

Cyp3A4 is known to be involved in the metabolism of more than half of all currently used drugs. Several well-documented cases exhibiting clinically important drug-drug interactions and toxicities are implicated to be related to Cyp3A4 inhibition. *Cyp3a11* and *Cyp3a13*, two important Cyp3A isoforms identified in the mouse, are expressed in differentiated ES cells (Fig. 4). Cytochrome P450 3A activity of ES cells differentiated for 60 days was tested (Fig. 5C).

ES cell-derived hepatic cells (d60) exhibited metabolic activities approximately one fifth of that of adult liver (AL). These data are consistent with real-time PCR results (Fig. 4).

### **Application to human ES cells**

To test if the present culture procedure could be applied to human ES cells, KhES-1 cells were treated at d0 to d10 with Activin and LY294002, a potent PI3 kinase inhibitor, which was shown to efficiently promote endoderm differentiation from undifferentiated ES cells (McLean *et al.* 2007). Since there is evidence for a role of the FGF signaling pathway in maintaining pluripotency of human ES cells (Amit *et al.* 2000), bFGF was not used in the present procedure for differentiation of human ES cells although it promoted endoderm differentiation in mouse ES cells.

Figure 6A shows the expression levels of the transcripts of endoderm or hepatic markers in human ES cell-derived differentiated cells (d18, d30). An endoderm marker, *Sox17* as well as *Afp*, *Alb*, *Cytokeratin 7 (CK7)*, *Cytokeratin 18 (CK18)*, *Cytokeratin 19 (CK19)* and *Cyp7a1* were detected in differentiated ES cells on d18. On d30, the expression of *Afp* decreased, along with a marked increase in *Alb* expression. ICC analyses demonstrate that AFP is induced in approximately 40% of total cells on d14 (Fig. 6B, Supplementary Table 2). Addition of Dex, HGF at d10 and thereafter increased the proportion of AFP-expressing cells with concomitant decrease in Oct3/4 expressing undifferentiated ES cells (Supplementary Table 2). Albumin positive cells are detected on d18, where there are cells expressing both AFP and Albumin (Fig. 6B). The proportion of AFP-expressing cells decreased much more slowly comparing with mouse ES cells (Supplementary Table 2). On d30, colonies of Albumin single positive cells, which did not express AFP, become evident (Fig. 6B). On d20, approximately 80% of the human ES cells differentiated into AFP-positive cells, and on d40, 9% of total cells were Albumin-positive hepatocytes. These results suggest that the human ES cell-derived hepatic cells differentiate sequentially to form hepatocyte precursor, hepatoblast, and then hepatocyte or

bile duct lineages similarly with that observed in mouse ES cells, but there were differences in the length of time window required for the process of maturation.

Next, to examine the extent of maturation of the human ES cell-derived hepatic cells, hepatic molecular markers were analyzed by quantitative PCR with d18 and d50 cultures (Fig. 7A). The expression levels of the molecular markers were compared with those in 22 – 40 weeks old fetal liver or adult liver. *Afp* was expressed on d18, and decreased on d50. The expression level on d50 was much higher than that of fetal liver. The mature hepatocyte markers such as *Albumin (Alb)*, *Cyp3a4*, liver-specific *organic anion transporting polypeptides 1B1 (Oatp1b1)* (Hsiang *et al.* 1999; Konig *et al.* 2000) were detected on d18 and increased to a substantial level on d50 (Fig. 7A). Most cells on d24 and d50 were PAS positive, demonstrating glycogen deposition (Fig. 7B).

Ultrastructures of the differentiated human ES cells grown for 50 days were evaluated by electron microscopy (Fig. 7C-E). ES cell-derived cells were rich in organelle such as mitochondria (Mt), endosome (En), lysosome (Ly), rough ER (rER), Golgi apparatus (Go), being consistent with typical hepatocytes with active protein synthesis (Fig. 7C-E). In addition, some of the cells formed biliary canaliculi (Bc) with microvilli associated with intracellular filament (Fi) and cell junctional complexes (arrows) (Fig. 7C). Consistent with results from PAS staining, most of ES cell-derived cells were rich in glycogen granules (g), and convoluted form of smooth endoplasmic reticulum (sER) were also found in the cytoplasm (Fig. 7D). Furthermore, some of these cells showed characteristic epithelial like structure with polarization (Fig. 7E); well-developed microvilli were prominent on the one side of the cell surface (Fig. 7E arrowhead), and cell junction complex also observed on the lateral side (Fig. 7E arrow). These results indicate that ES cell-derived hepatic cells show normal hepatocyte ultrastructure.

## DISCUSSION

We previously reported a novel procedure for *in vitro* differentiation of ES cells into definitive endoderm lineages by growing ES cells on a supporting cell line, M15, and that by modification of the culture condition, the differentiation efficiency of ES cell into Pdx1-expressing cells markedly increased (Shiraki *et al.* 2008). The molecular mechanism for each inductive process during the entire differentiation procedure was extensively studied (Shiraki *et al.* 2008). With this information, we are able to optimize the condition for selective induction of hepatic fate versus a pancreatic fate of the definitive endoderm at a high efficiency (Fig. 1). Several groups have recently reported the differentiation of ES cells into hepatic cells (Gouon-Evans *et al.* 2006; Soto-Gutierrez *et al.* 2006; Cai *et al.* 2007). However, this is the first report demonstrating the expression of mature hepatocytes markers of various transporters and metabolizing enzymes, such as *Sult2a1*, *Ugt1a1*, *Cyp7a1*, *Cyp2b10*, *Cyp3a11*, *Cyp3a13*, *Slco1a4*, *Abcb11* (Fig. 4). Moreover, under present culture condition, the mature hepatocytes can be maintained over 60 days *in vitro* (Fig. 5).

During mouse development, the induction of hepatic genes occurs about E8.5 (Lemaigre & Zaret 2004), and it requires FGF signaling from adjacent cardiac mesoderm (Jung *et al.* 1999) and BMP2&4 from the septum transversum mesenchyme cells (Rossi *et al.* 2001). Then the endoderm starts to bud out into the stromal environment, where they interact with endothelial cells (Matsumoto *et al.* 2001). When the hepatic endoderm is specified, they are referred to as hepatoblasts (Lemaigre & Zaret 2004). The bipotential hepatoblasts are capable of differentiating into hepatocytes or bile duct cells. Our results suggest that hepatic differentiation is enhanced under serum free condition which agrees with previous reports (Kubo *et al.* 2004; Heo *et al.* 2006) that serum inhibits hepatic differentiation. It is also demonstrated that removal of serum strongly enhanced hepatic induction while inhibiting Pdx1 expression, indicating that there were unknown serum factors inhibiting hepatic differentiation (Fig. 1 and 2). We found that the treatment of Dex, HGF and OsM under serum containing conditions elevated

the expression level of *Shh*, but not *Afp* in mouse ES cells (Fig, 1B). *Shh* is expressed in the foregut but not pancreatic or hepatic epithelia. Therefore these results suggested that endoderm-derived cells other than pancreas and liver were differentiated under the above culture condition. Taken together, our differentiation systems using M15 provide a good tool for investigation of regionalization of gut endoderm *in vitro*.

We also examined the effects of BMP, bFGF and their antagonists, Noggin or SU5402 (Fig. 2A, B). While bFGF increased AFP-expressing cells, SU5402 decreased. Noggin completely abolished AFP-expressing cells, and this was rescued by bFGF, demonstrating that BMP4 and bFGF are both required for hepatic differentiation. The addition of BMP did not show potentiation effects on the formation of AFP positive cells in this study (Fig. 2C, D). We speculated that endogenous BMP secretion from M15 cells, in which *Bmp4* is highly expressed (Shiraki et al. 2008), is sufficient. The above results agree with a recent report that *in vitro* generation of ES cell derived hepatic cells requires BMP4 (Gouon-Evans et al. 2006), and are consistent with previous reports using mouse embryo (Jung et al. 1999; Rossi et al. 2001), thereby suggesting that *in vitro* hepatic differentiation using our method recapitulates the mouse hepatic development.

HGF has been reported as a regulator of hepatoblast bipotency (Suzuki et al. 2002; Suzuki et al. 2003). Dexamethasone is a synthetic glucocorticoid hormone active in induction of enzymes concerned in gluconeogenesis in the liver (McGrane et al. 1990; Michalopoulos et al. 2003). In our present studies, HGF and dexamethasone promoted expression of AFP and Albumin (Fig. 2). *Afp* transcription was reported to start soon after fertilization, whereas the *Alb1* transcripts was detected by RT-PCR, at the earliest, on E13.5 (Petkov et al. 2004). Our present results show a temporally correlated expression pattern of *Afp* and *Alb* (Fig. 3) thereby indicating recapitulation of normal liver embryonic developmental program. DBA lectin-positive bile duct cells were also generated from ES cells, indicating that the present procedure could induce cells of the hepatic lineages, including bile duct cells and hepatocytes

(Fig. 3). Enzymes function in the detoxification pathway; such as *Sult2A*, *Ugt1A*, *Cyp2b10*, *Cyp3a11* and *Cyp3a13*, are induced (Fig. 4). Biochemical analyses indicated that ES cell-derived hepatocytes deposited glycogen, which are characteristics of normal mature hepatocytes (Fig. 5A).

On the other hands, most of the genes expressed in the hepatic cells are also expressed in the extra embryonic yolk sac tissue. AFP and Albumin, which are widely used as hepatic marker, are also expressed in both tissues. In order to discriminate between these two populations of cells, ICC of Cyp7a1, a hepatocyte specific marker which is not expressed in the extraembryonic yolk sac tissue, was performed. As shown in Figure 3C and 6A, both mouse and human ES cell-derived differentiated cells expressed Cyp7A1, thereby suggesting that Albumin-positive cells derived from ES cells are hepatic cells but not extra embryonic cells.

Maturation of the liver is reported to be governed by cytokine signaling (Matsumoto *et al.* 1994; Kinoshita & Miyajima 2002). Studies using hepatoblast culture system have shown that OsM, which is secreted from hematopoietic cells within the fetal liver, contributes to control late stages of hepatocyte differentiation (Kamiya *et al.* 1999; Kamiya & Gonzalez 2004). Albumin secretion assay suggests that OsM promotes hepatic maturation in our differentiation systems (Fig. 5B). Finally ES cell-derived hepatic cells grown with Dex, HGF and OsM for 60 days exhibit some extents of drug metabolism activity (Fig. 5C).

A human ES cell line, KhES-1 (Suemori *et al.* 2006), was also subjected to the similar procedure as mouse ES cells and cultured on M15 cells as a supporting cell. KhES-1 gave rise to abundant AFP-positive cells, and by combination of growth factors, they expressed AFP and Albumin in a sequential way similar to that of mouse ES cells. This suggests that KhES-1 cells adopt similar pathway toward hepatocytes (Fig. 6A, B). Additionally, the results of PAS staining, quantitative PCR analysis and transmission electron microscopy suggest that human ES cell-derived cells have the characteristics of mature hepatocytes (Fig. 7).

In conclusion, the use of M15 as supporting cells for the differentiation of ES cells has been

proven to be a powerful tool for regulating ES cell differentiation especially into endodermal fate. The differentiation process recapitulates normal development, thus helps understanding the molecular mechanism of the processes of induced differentiation of cells of the endoderm lineages. The applicability of this method to human ES cells would yield the potential tools for drug discovery or regenerative medicine in the future.



## EXPERIMENTAL PROCEDURE

### *ES cell lines*

The mouse ES cell line, SK7, containing a *Pdx1* promoter-driven GFP reporter transgene, was established by culturing blastocysts obtained from transgenic mice homozygous for the *Pdx1*/GFP gene (Shiraki *et al.* 2008). The SK7 ES cell line was maintained on mouse embryonic fibroblast (MEF) feeders in Glasgow minimum essential medium (Invitrogen) supplemented with 1000 units/ml leukemia inhibitory factor (LIF; Chemicon), 15% Knocked-out Serum Replacement (KSR; Gibco), 1% fetal bovine serum (FBS; Hyclone), 100  $\mu$ M nonessential amino acids (NEAA; Invitrogen), 2 mM L-glutamine (L-Glu; Invitrogen), 1 mM sodium pyruvate (Invitrogen), 50 units/ml penicillin and 50  $\mu$ g/ml streptomycin (PS; Invitrogen) and 100  $\mu$ M  $\beta$ -mercaptoethanol ( $\beta$ -ME; Sigma). The human ES cells (KhES-1) (Suemori *et al.* 2006) were a gift from Dr. N. Nakatsuji and Dr. H. Suemori (Kyoto University, Kyoto, Japan) and were used following the hES cell guidelines of the Japanese government. Undifferentiated hES cells were maintained on a feeder layer of MEF in DMEM/F12 (Sigma) supplemented with 20% KSR, L-Glu, NEAA and  $\beta$ -ME under 3% CO<sub>2</sub>. To passage hES cells, hES cell colonies were detached from the feeder layer by treating them with 0.25% trypsin and 0.1 mg/ml collagenase IV in PBS containing 20% KSR and 1 mM CaCl<sub>2</sub> at 37°C for 5 min, followed by adding culture medium and disaggregating ES cell clumps into smaller pieces (5-20 cells) by gentle pipetting several times.

### *Supporting cells*

The mesonephric cell line M15, which expresses WT1, was established from mouse mesonephros overexpressed with the large T protein of polyoma virus under the control of the early viral enhancer. M15 cells (Larsson *et al.* 1995) was kindly provided by Dr. T. Noce (Mitsubishi Kagaku Institute of Life Science, Tokyo, Japan) and Dr. M. Rassoulzadegan (University of Nice-Sophia Antipolis, Antipolis, France). M15 cells (Larsson *et al.* 1995) were

grown in DMEM supplemented with 10% FBS. M15 cells were treated with mitomycin C at 10 µg/ml for 2.5 hr before use. M15 were seeded on gelatin-coated 6-well or 24-well plates at a concentration of  $8 \times 10^5$  cells or  $2 \times 10^5$  cells per well, respectively.

### ***Growth factors and inhibitors***

Reagents were purchased and used at the designated concentrations as followings; recombinant human activin-A (R&D Systems), 20 ng/ml; recombinant human bFGF (Peprotech), 50 ng/ml; recombinant human BMP4 (R&D Systems), 50 ng/ml; LY294002 (Calbiochem), 10 µM; SU5402 (Calbiochem), 10 µM; recombinant mouse Noggin/Fc Chimera (R&D Systems), 100 ng/ml; recombinant human Hepatocyte Growth Factor (HGF, Peprotech), 10 ng/ml; Dexamethasone (Dex, Sigma), 1 µM; recombinant human Oncostatin M (OsM, Sigma).

### ***Differentiation of ES cells***

For differentiation studies, ES cells were plated at 5,000 or 20,000 cells per well in 24-well or 6-well plates (Nunc), respectively, that had been previously coated with M15 cells. The cells were cultured in differentiation medium supplemented with 10%FBS and 4500 mg/L glucose on d0 - d4, and were switched to 10%KSR and 2000 mg/L glucose at d4 and on, in DMEM with NEAA, L-Glu, PS and β-ME for up to 60 days. Activin A (20 ng/ml) and bFGF (50 ng/ml) were added on d0 - d4 of differentiation (Shiraki *et al.* 2008), withdrawn and switched to as noted in the text on d4 and on. Media were replaced every 2 days with fresh medium supplemented with growth factors.

In the case of human ES cells, KhES-1 cells were plated at 20,000 or 80,000 cells per well in 24-well or 6-well plates, respectively, that had been plated with M15 cells. ES cells were cultured in differentiation medium (DMEM supplemented with 10% KSR, 4500 mg/L glucose, NEAA, L-Glu, PS and β-ME) for up to 50 days. Activin A (20 ng/ml) and LY294002 (10 µM) were added on d0-d10 of differentiation, HGF (10 ng/ml) and Dex (1 µM) were added on d10 -

d50. Media were replaced every 2 days with fresh differentiation medium supplemented with growth factors.

### ***Flow cytometry analysis***

Either of the following antibodies were used: biotin-conjugated anti-E-cadherin monoclonal antibody (mAb) ECCD2 (Shirayoshi *et al.* 1986), biotin-conjugated anti-CD45 mAb B220 (eBioscience), biotin-conjugated anti-TER-119 mAb (eBioscience) or phycoerythrin (PE)-conjugated anti-Cxcr4 mAb 2B11 (BD Biosciences Pharmingen). The stained cells were analyzed with a FACS Canto (BD) or purified with FACS Aria (BD). Data were recorded with the BD FACS Diva Software program (BD) and analyzed using the Flowjo program (Tree Star).

### ***RT-PCR analysis***

RNA was extracted from ES cells or mouse liver using RNeasy mini-kit (Qiagen) and then treated with DNase (Qiagen). Human fetal (22-40 weeks old) and adult (51 years old) liver total RNA were purchased from Clontech Laboratories, Inc. For reverse transcription reactions, 3 µg RNA was reverse-transcribed using MMLV reverse transcriptase (Toyobo) and oligo dT primers (Toyobo). One µl of 5-fold diluted cDNA (1% of the RT product) was used for PCR analyses. The primer sequences for each primer set are shown in Supplementary Table 1 in the supplementary material. For real-time PCR analysis, the mRNA expression was quantified with SyberGreen on an ABI 7500 thermal cycler (Applied Biosystems). The level of each gene expression was normalized with that of *β-actin*. The PCR conditions were as follows: denaturation at 95°C for 15 sec, annealing and extension at 60°C for 60 sec, for up to 40 cycles. Each measurement were normalized to *β-actin* (mouse) and *GAPDH* (human) for each sample by subtracting the average *β-actin* (mouse) and *Gapdh* (human) Ct values (Threshold Cycle) from the average Each gene Ct, resulting in Ct. Each target mRNA levels, expressed as arbitrary units, were determined by standard curve method.

### ***Immunocytochemistry***

For whole-mount immunocytochemistry, ES cells were plated on 24-well Thermanox cover slips (Nunc). Confocal images were obtained using a TCS-SP2 Spectral Confocal Scanning System (Leica). Phase contrast images were obtained using IX71 Inverted Microscope (Olympus). The following antibodies were used to detect the following: mouse anti-GFP (Roche), rabbit anti- $\alpha$ -fetoprotein (AFP, Biomeda), goat anti-Albumin (Sigma), fluorescein isothiocyanate (FITC)- conjugated *Dolichos biflorus agglutinin* (DBA) lectin (Sigma), rabbit anti- $\alpha$ -1-Antitrypsin (Sigma), rabbit anti-CYP3A2 (Biomol), rabbit anti-CYP7A1 (Santa Cruz), mouse anti-Oct3/4 (Santa Cruz) Secondary antibodies used were Alexa 568-conjugated goat anti-rabbit, donkey anti-goat antibodies (Molecular Probes); Alexa 488-conjugated goat anti-mouse and anti-rabbit, donkey anti-goat antibodies (Molecular Probes). Cells were counterstained with DAPI (Roche). The numbers of stained cells in Figure 3 were quantified by analyzing, the areas of AFP-positive staining images using Lumina Vision program (Mitani Corporation) (Supplementary Table 2)

### ***Period acid Schiff (PAS) analysis***

The cultured cells were fixed in 3.3% formalin for 10 min, and intracellular glycogen was stained using a PAS staining solution (Muto Pure Chemicals), according to the manufacturer's instructions.

### ***Albumin secretion assay***

The culture media were replaced with fresh media and the conditioned media were harvested 24 hours later and were assayed for Albumin secretion using the Lebis ELISA kit (Shibayagi).

### ***Assay for cytochrome P450 3A activity***

To confirm cytochrome P450 3A activity, 3µg samples of microsomes were analyzed using the P450-Glo™ CYP3A4 Assay Kit (Promega). Microsomes were prepared from livers of 6-week-old male ICR mice, undifferentiated ES cells, and differentiated ES cells. The protein content of the microsome preparations was measured using the Micro BCA™ Protein Assay Kits (Pierce).

### ***Transmission Electron Microscopy***

Human ES cell-derived hepatic cells grown on M15 in tissue culture plates for 50 days were fixed with 2% glutaldehyde-cacodylate buffer for 30 min, postfixed with 1% osmium tetroxide for 30 min, after which they were dehydrated with graded series of ethanol. Samples were embedded in Epok 812 (Oken Trading, Tokyo, Japan), and ultrathin sections were cut with an ultramicrotome (MT7000-Ultra; RMC, Tucson, AZ, USA). Samples were stained with uranyl acetate and lead citrate and were then viewed with an electron microscope (H-7500; Hitachi, Tokyo, Japan).

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## FIGURE LEGENDS

### Figure 1

**Modification of the previous differentiation protocol of ES cells results an efficient differentiation of mouse ES cells into *Afp* expressing hepatic endoderm lineages at the expense of *Pdx1*-expressing cell lineages.**

(A) Quantification by flow cytometry analysis of gut definitive endoderm (E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup>) or *Pdx1*/GFP expressing cells. ES cell were differentiated on M15 cells, added with Activin and bFGF at d0-d4. Then at d4-d8, ES cells were cultured at a combination of various soluble factors, in media supplemented with FBS or KSR with varied glucose concentration (2000 or 4500 mg/L) and were evaluated on d8, for the proportion of definitive endoderm cells defined by E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup>, or *Pdx1*-expressing cells using flow cytometry analysis. (B) Relative *Shh* and *Afp* transcript levels within E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup> definitive endoderm in the differentiated ES cells by real-time PCR analysis. Gut definitive endoderm (E-cadherin<sup>+</sup>/Cxcr4<sup>+</sup>) cells were separated using flow cytometry. cDNA from these definitive endoderm cells were used as templates. *Shh* and *Afp* transcript levels were quantified and normalized against those of  $\beta$ -actin. The results are compared with that of controls in 4500FBS (the differentiated ES cells grown under conditions with 4500mg/L glucose, 10%FBS and without growth factors), which is defined as 1. (A, B) Control (white bars; without growth factors), Activin&bFGF (black bars; with 20 ng/ml Activin and 50ng/ml bFGF), Dex&HGF&OsM (gray bars; with 1  $\mu$ M dexamethasone, 10 ng/ml HGF and 10ng/ml Oncostatin M). 2000FBS (2000mg/L glucose, 10%FBS), 2000KSR (2000mg/L glucose, 10%KSR), 4500FBS (4500mg/L glucose, 10%FBS), 4500KSR (4500mg/L glucose, 10%KSR).

**Figure 2 Effects of various factors on the formation of ES cell-derived *Pdx1*/GFP- or AFP-expressing cells were compared by immunocytochemical analysis.**

(A) ES cells cultured for 8 days were stained for AFP (red) and GFP (*Pdx1*; green). The effects of various factors added on d4-d8 are compared. ES cells were differentiated on M15 cells, added with Activin & bFGF on d0-d4. On d4-d8, Activin & bFGF were withdrawn and the following culture conditions were tested: control (without any factors); Activin & bFGF (with Activin and bFGF), Dex & HGF & OsM (with Dex, HGF and OsM), and cultured in medium with KSR or FBS, at a glucose concentration at 2000 or 4500 mg/ml (2000FBS, 2000KSR, 4500FBS, 4500KSR). Bars indicate 200  $\mu$ m. (B) Quantitative analysis of the effects of Dex, HGF and OsM on hepatic differentiation. On d4-d8, addition of one, or combinations of two factors, or all three factors were added to the ES cell cultures in 2000KSR condition. Fold increases in AFP-positive cells compared to control. AFP-positive area was analyzed using a Lumina Vision program. (C, D) BMP and FGF signals are pivotal for hepatic differentiation of the definitive endoderm from ES cells. ES cells were allowed to differentiate with Activin and bFGF in media supplemented with Activin and bFGF, 10% FBS and 4500 mg/L glucose on d0-d4, then switched to 10% KSR and 2000 mg/L glucose, with or without the indicated growth factors. (C) ICC analysis using anti-AFP antibodies, with DAPI counterstained the nuclei. The addition of Noggin inhibited differentiation into AFP-positive cells, but bFGF addition rescued the inhibitory effect of Noggin. Bars indicate 200  $\mu$ m. (D) Fold increase in AFP positive cells compared to control. AFP positive region in each fluorescence image was analyzed using Lumina Vision program. Values represent means  $\pm$  standard error of the mean (n=3). \* $p < 0.05$  and \*\* $p < 0.01$  vs control by Student's *t*-test.

**Figure 3 Chronological analysis revealed a sequential induction of hepatic cells recapitulating normal hepatic development.**

ES cells were differentiated at d0-d4 in media supplemented with Activin and bFGF, 10% FBS and 4500 mg/L glucose. At d4-d8, the supplements were switched to Dex, and HGF, 10% KSR and 2000 mg/L glucose. (A) Differentiated ES cells on d10, d18, d22 and d30 were stained for AFP (green) and Albumin (red). On d10, AFP+ hepatoblast like cells arise (d10, green). AFP+/Albumin+ cells appeared at d18 of differentiation (d18, merged as yellow). On d22, AFP-/Albumin+ mature hepatocytes increased (d22, red), while AFP+ cells (d22, green) reduced in number. On d30, AFP+ cells almost disappeared and Albumin+ cells form distinct colonies. (B) Differentiated ES cells on d30 were stained for Albumin (a hepatocyte marker, red) and *Dolichos biflorus* agglutinin (DBA) lectin (a bile duct marker, green). Albumin+ and DBA lectin+ cells were detected in colonies distinct from each other. (C) Differentiated ES cells on d30 were stained for Albumin (green) and mature hepatocyte markers (red):  $\alpha$  1-Antitrypsin, Cyp3A and Cyp7A1. Bars indicate 200  $\mu$ m.

**Figure 4 Molecular analysis of hepatic molecular markers by real-time PCR of ES cell-derived hepatocytes compared with those from fetal liver or adult liver.**

Transcripts of hepatic marker genes were quantified by real-time PCR analyses. ES cells were differentiated on d0-d4 in media supplemented with Activin and bFGF, 10% FBS and 4500 mg/L glucose. On d4-d30, the supplements were switched to Dex, and HGF, 10% KSR and 2000 mg/L glucose. The transcript level was normalized with that of  *$\beta$ -actin*. Each value is normalized with that of the differentiated ES cells on d30 (d30) and thus the graphs represent relative gene expression level when the level on d30 is defined as 100. ES cells were cultured on optimal condition for hepatic differentiation, with Dex and HGF. M15; M15 cells, ES; undifferentiated ES cells, d10 and d30; differentiated ES cells grown on M15 cells at d10 and d30, FL; CD45-/Ter199- E12.5 fetal liver cells, AL; adult liver.

### **Figure 5 Analyses of ES cell-derived hepatocytes.**

ES cells were cultured on optimal condition for hepatic differentiation, with OsM. ES cells were differentiated on d0-d4 in media supplemented with Activin and bFGF, 10% FBS and 4500 mg/L glucose. (A) On d4-d30, the supplements were switched to Dex, and HGF, 10% KSR and 2000 mg/L glucose (Dex&HGF/2000KSR). (B) On d4-8; Dex&HGF/2000KSR, on d8-60; Dex&HGF/2000KSR or Dex&HGF&OsM/2000KSR. (C) On d4-8; Dex&HGF/2000KSR, on d8-60 Dex&HGF&OsM/2000KSR. (A) Periodic acid Schiff assay performed on d30 differentiated ES cells indicates numerous hepatocytes within the colonies with cytoplasm glycogen storage (dark red), whereas not detected in differentiated ES cells on d4 or M15 cells. (B) Albumin-secreting potential of ES cell-derived hepatic cells was assayed with ELISA. The differentiation medium was changed to fresh medium 24h before assay. The amount of albumin released from the ES cell-derived hepatic cells into the medium per 24 hours was measured in each medium on d8, d40, d50 and d60 of differentiation. (C) Cytochrome P450 3A4 activity of ES cell-derived hepatic cells. Each value is normalized with the amount of protein and thus the graphs represent relative activity when the activity of adult liver is defined as 1. ES; undifferentiated ES cells, d60; differentiated ES cells grown on M15 cells on d60, AL; adult liver. Values represent means  $\pm$  standard error of the mean (n=3).

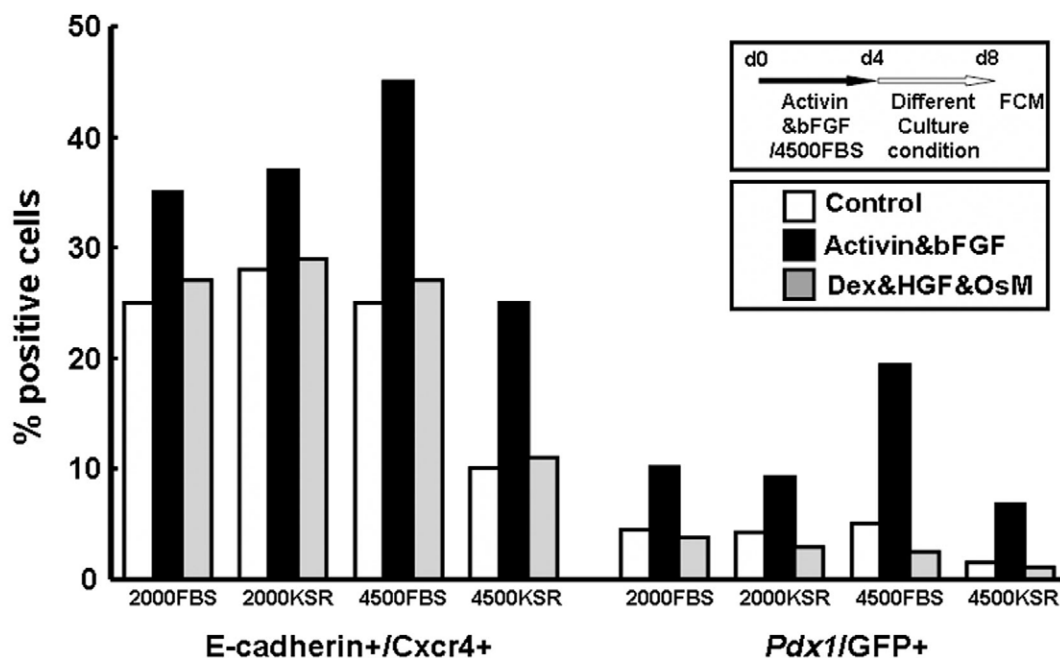
### **Figure 6 Differentiation of human ES cells into hepatic lineages**

Differentiated human ES cells (KhES-1) on d10, grown on M15 cells, supplemented with 20 ng/ml Activin, 10 $\mu$ M LY294002, 10% KSR and 4500 mg/L glucose. On d10, supplements were switched to 1  $\mu$ M Dex and 10 ng/ml HGF. (A) RT-PCR analysis of endoderm or hepatic markers in differentiated ES cells. hES; undifferentiated human ES cells, d18 and d30; differentiated human ES cells grown on M15 cells at d18 and d30, M15; M15 cells. (B) Differentiated ES cells on d14, 18 and 30 were stained for AFP (green) and Albumin (red).

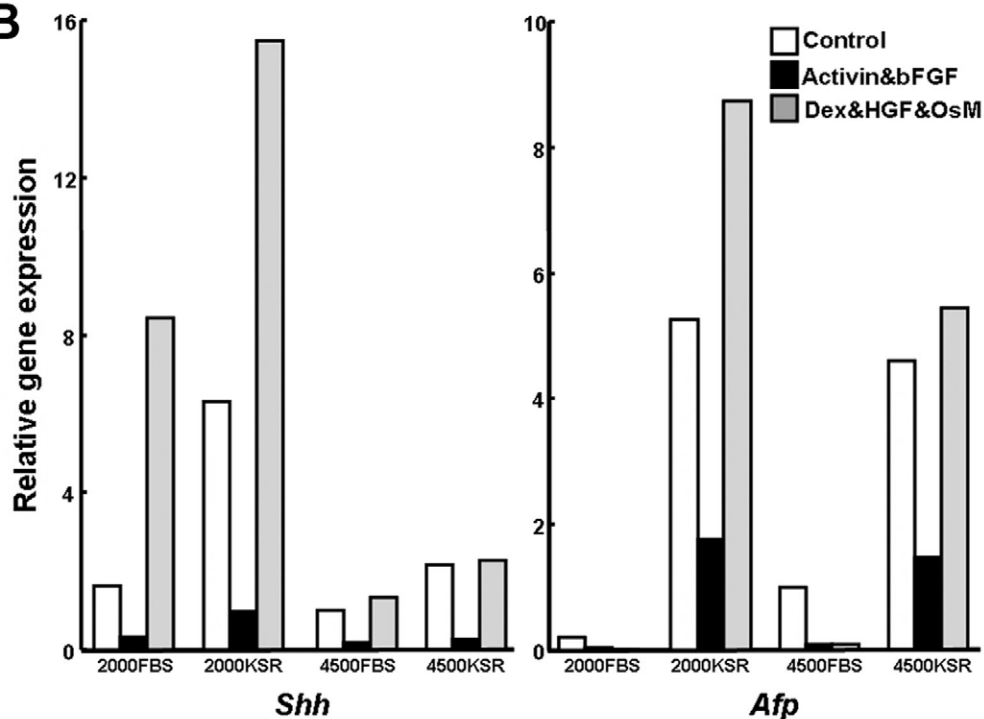
### **Figure 7 Analyses of human ES cell-derived hepatocytes.**

(A) Transcripts of hepatic marker genes were quantified by real-time PCR analyses. The transcript level was normalized with that of *Gapdh*. Each value is normalized with that of the differentiated ES cells on d50 (d50) and thus the graphs represent relative gene expression level when the level on d50 is defined as 100. hES; undifferentiated human ES cells, d18 and d50; differentiated ES cells grown on M15 cells on d18 and d50, FL; human fetal liver (22-40 weeks old), AL; human adult liver (51 years old). (B) PAS staining performed on d24 and 50 differentiated ES cells indicates numerous hepatocytes within the colonies with cytoplasmic glycogen storage (dark red). Bars indicate 200  $\mu$ m. (C-E) Transmission electron microscopy of ES-derived differentiated cells on d50. Micrograph of hepatocytes derived from human ES cells indicated hepatocyte ultrastructure described in the text. Notes, Bc, bile canaliculus, En, endosome, Fi, intracellular filament, g, glycogen, Go, Golgi apparatus, Ly, lysosome, Mt, mitochondria, N, nucleus, sER, smooth endoplasmic reticulum and rER, rough endoplasmic reticulum. The arrows in C and E indicated cell junction complexes, and arrowheads in F indicate microvilli. Scale bars in C, D, and E were 6.7  $\mu$ m, 667 nm, 2  $\mu$ m, respectively.

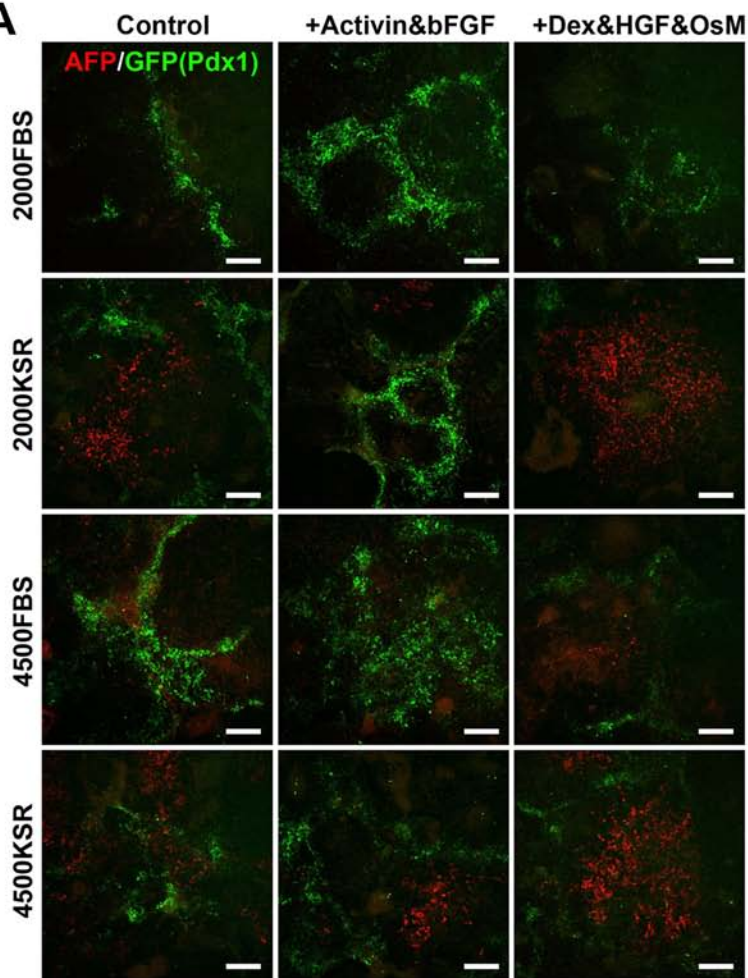
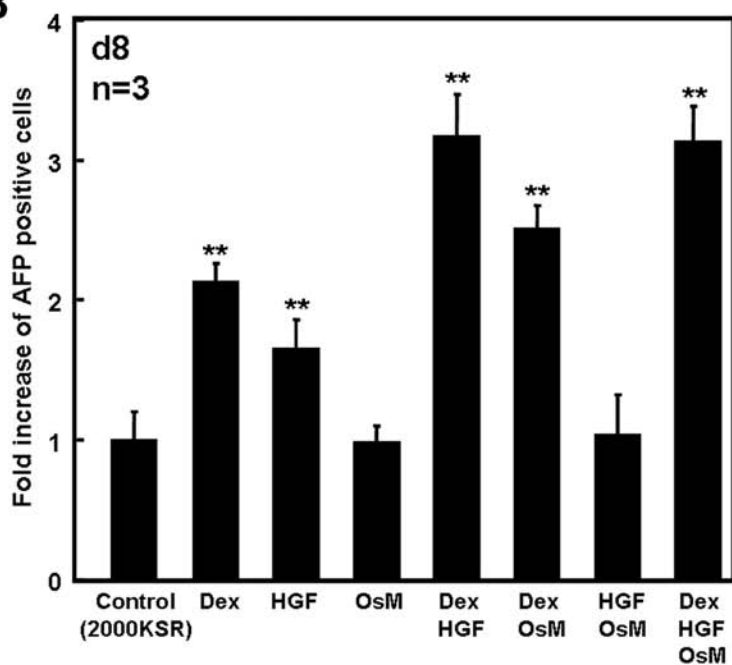
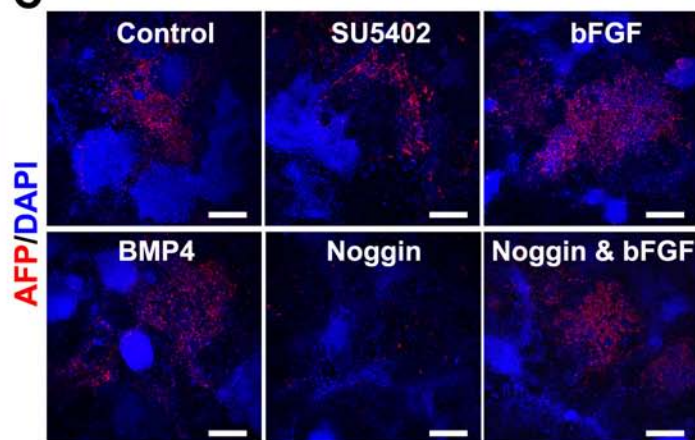
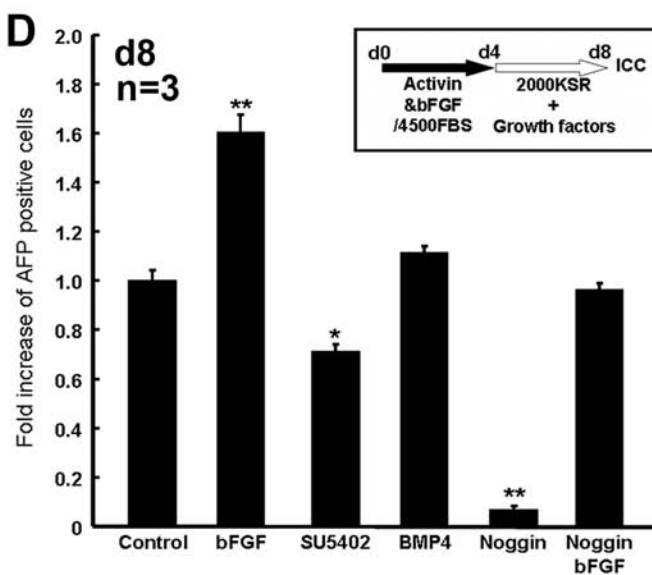
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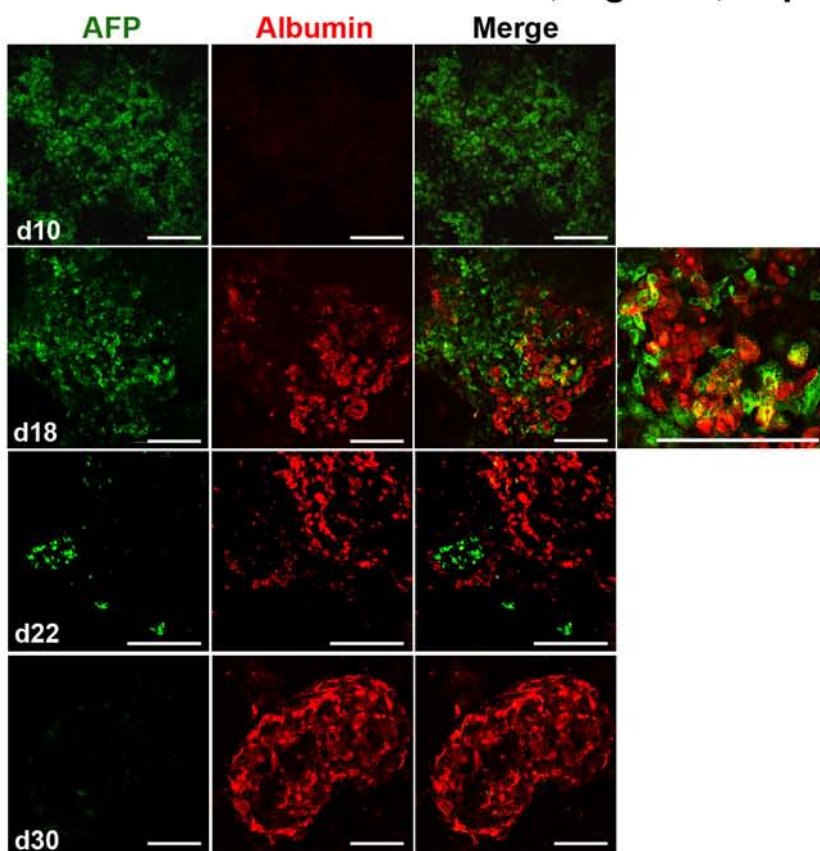
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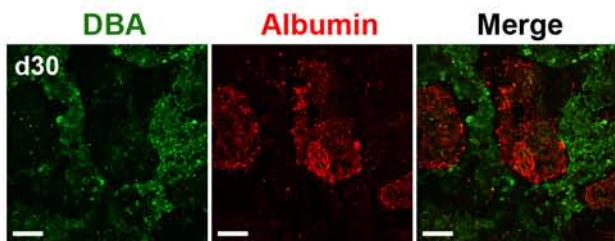


**A**

**B**

**C**

**D**


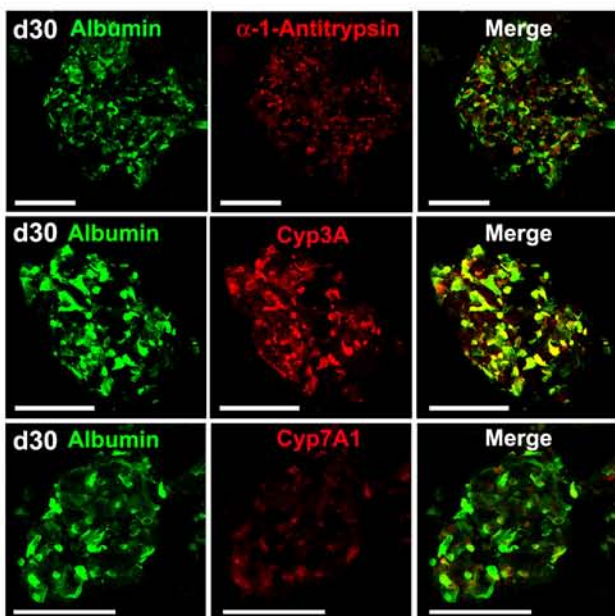
**A**



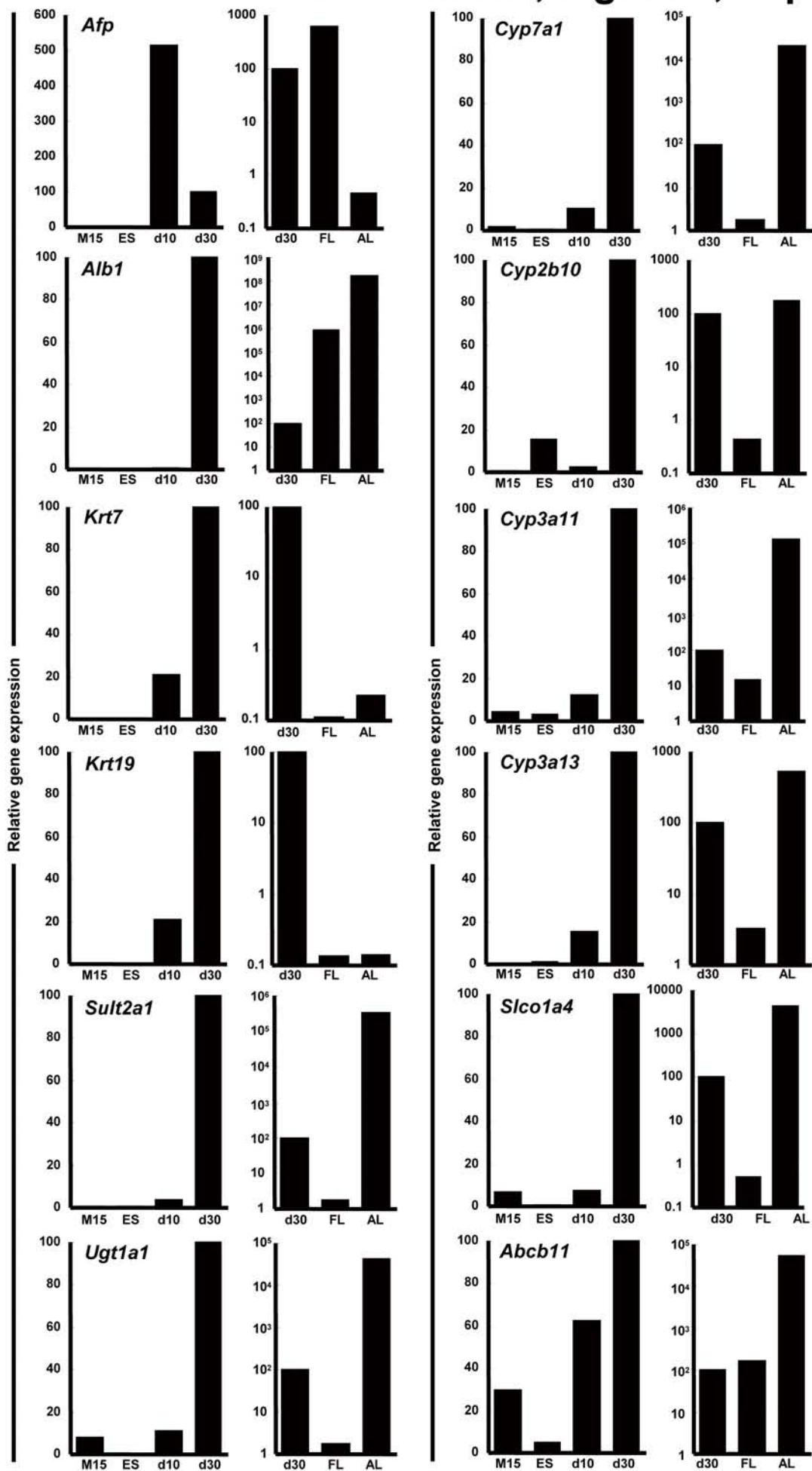
**B**



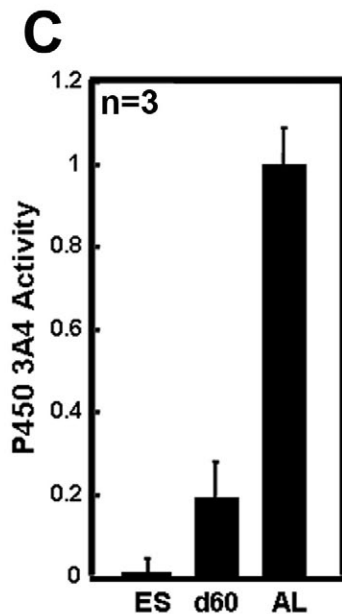
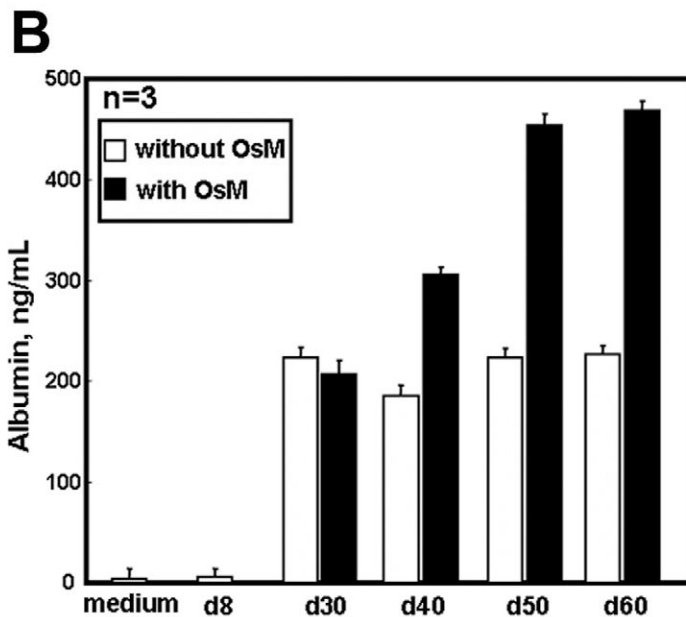
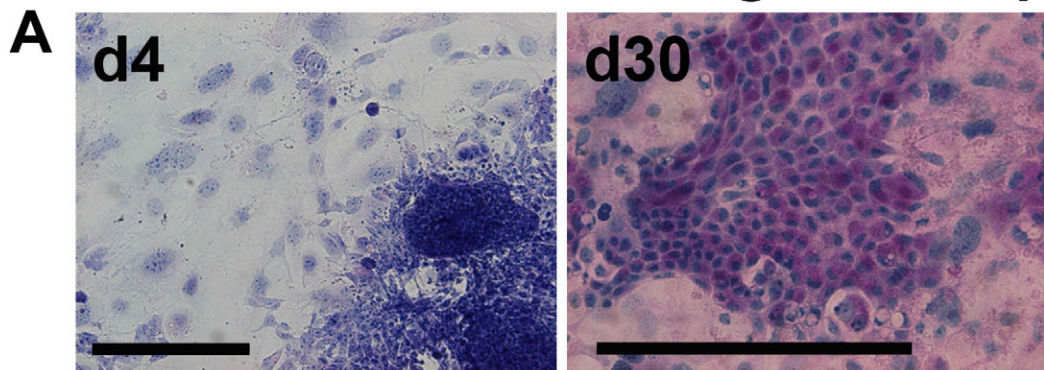
**C**



# Shoen Kume, Figure 4, Top

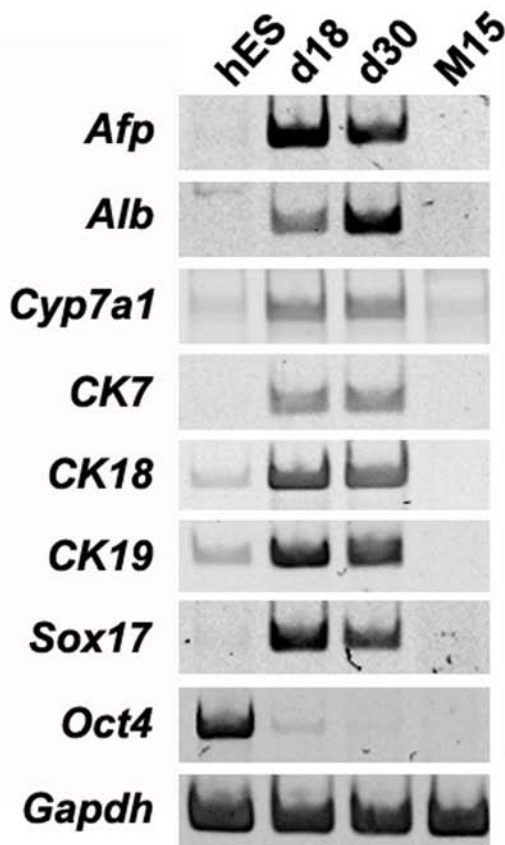


# Shoen Kume, Figure 5, Top

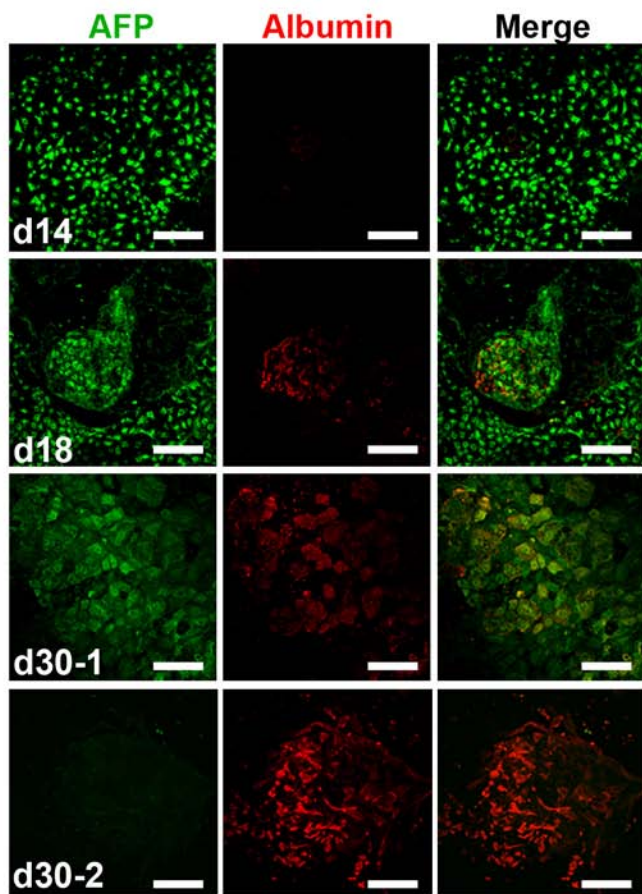


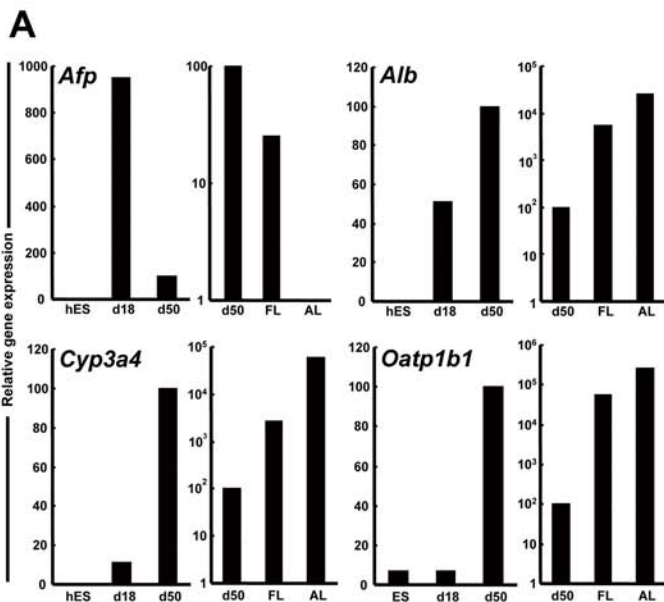


**A**

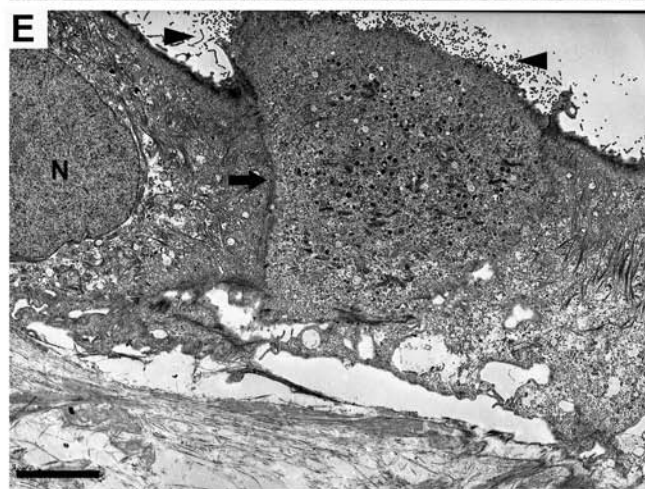
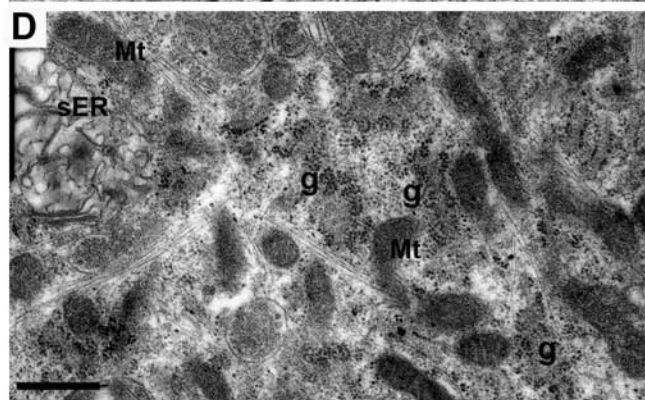
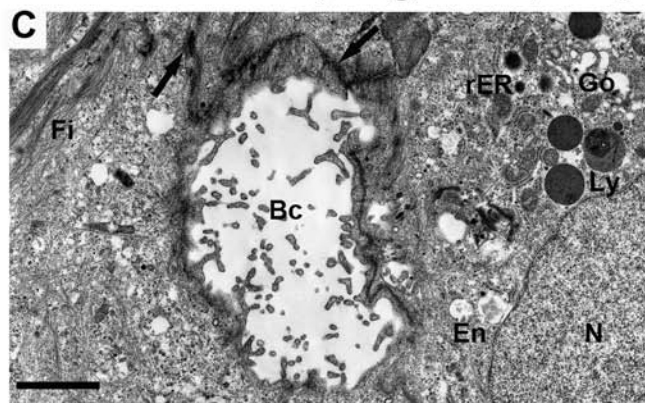
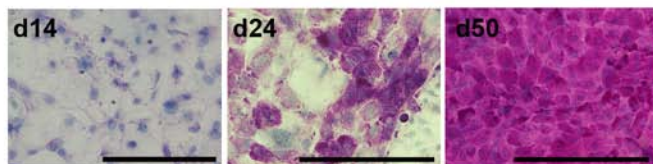


**B**





**B**



**Table 1. PCR primers used to detect mouse gene expressions**

Gene	Forward primer	Reverse primer
<b><i>mouse</i></b>		
<i>Abcb11 (Bsep)</i>	GAGTGGTGGACAGAAGCAAA	TGAGGTAGCCATGTCCAGAA
<i>Afp</i>	TCGTATTCCAACAGGAGG	AGGCTTTTGCTTCACCAG
<i>Alb1</i>	CTTAAACCGATGGGCGATCTCACT	CCCCACTAGCCTCTGGCAAAAT
<i><math>\beta</math>-actin</i>	GTGATGGTGGGAATGGGTCA	TTTGATGTCACGCACGATTTC
<i>Cyp2b10</i>	GCCCAATGTTTAGTGAGGA	GACTTCTCCTTCTCCATGCG
<i>Cyp3a11</i>	ATAGAGCTTTGTGTGCCCCC	CGGCTTTCCTTCATTCTGTC
<i>Cyp3a13</i>	CCCTGCTGTCTCCAACCTT	TGCGATTCTCTTTCAITCGTT
<i>Cyp7a1</i>	CAACGGGTTGATTCCATACC	ATTTCCTCATCAGTTTGACAG
<i>Krt7</i>	GGCGCTGGACATTGAGA	CCATGGTTCTCCGAAGATA
<i>Krt19</i>	GTCCTACAGATTGACAATGC	CACGCTCTGGATCTGTGACAG
<i>Pdx1</i>	CCAAAACCGTCGCATGAAGTG	CTCTCGTGCCCTCAAGAATTTTC
<i>Slco1a4 (Oatp2)</i>	GACGGCTCAGTGTTCACTC	CTTCTAGCTGGTCCCTCTT
<i>Sult2a1</i>	GGAAGGACCACGACTCATAAC	GATTCTTCACAAGGTTTGTGTTACC
<i>Ugt1a1</i>	TCTGAGCCCTGCATCTATCTG	CCCCAGAGGCGTTGACATA
<b><i>human</i></b>		
<i>Afp</i>	TGCCAACTCAGTGAGGACAA	TCCAACAGGCCTGAGAAATC
<i>Alb</i>	GATGTCTTCCTGGGCATGTT	ACATTGTGCTGCCCCACTTTTC
<i>CK7</i>	TCGCTGAGGTCAAGGCACAG	CACTCCTCAGCCTCGGCAAT
<i>CK18</i>	CCATGCGCCAGTCTGTGGAG	GTGGTGCTCTCCTCAATCTGCT
<i>CK19</i>	TGAGGTCATGGCCGAGCAGAAC	CATGAGCCGCTGGTACTCCTGA
<i>Cyp3a4</i>	CAGGAGGAAATTGATGCAGTTTT	GTCAAGATACTCCATCTGTAGCACAGT
<i>Cyp7a1</i>	AATCCATACCTGGGCTGTG	AGGCAGCGGTCTTTGAGTTA
<i>Gapdh</i>	CGAGATCCCTCCAAAATCAA	CATGAGTCCTTCCACGATACCAA
<i>Oatp1b1</i>	TGAACACCGTTGGAATTGC	TCTCTATGAGATGTCACCTGGAT
<i>Ocr4</i>	AGGTGTGGGGGATTCCCCCAT	GCGATGTGGCTGATCTGCTGC
<i>Sox17</i>	ACTGCAACTATCCTGACGTG	AGGAAATGGAGGAAGCTGTT
<i>ABcb11</i> , ATP-binding cassette sub-family B member 11; <i>Afp</i> , $\alpha$ -fetoprotein; <i>Alb1</i> , albumin1; <i>Krt7</i> (CK7), cytokeratin 7; <i>CK18</i> , cytokeratin 18; <i>Krt19</i> (CK19), cytokeratin 19; <i>Pdx1</i> ; pancreatic and duodenal homeobox 1, <i>Sult2a1</i> , sulfotransferase family 2A dehydroepiandrosterone-preferring member 1; <i>Ugt1a1</i> , UDP glucuronosyltransferase 1 family polypeptide A1		

**Table 2. Immunocytochemical analysis of protein expression in ES cell derived hepatic cells**

Antigen	Mouse ES (%)		Antigen	Human ES (%)		
	d8	d30		d14	d20	d40
AFP	33.1±1.7	7.4±0.9	Oct3/4	8.2±0.9	0	0
Albumin	0	17.8±1.3	AFP	40.4±3.6	79.4±2.8	58.5±5.1
DBA	0	72.9±3.8	Albumin	0	0.8±0.1	9.0±1.2

Data represent mean ±SEM of three samples.